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Alteration of Cell Cycle Kinase Complexes in Human Papillomavirus E6- and E7-Expressing Fibroblasts Precedes Neoplastic Transformation

YUE XIONG,[1,2,3,4] DHANDAPANI KUPPUSWAMY,[5]† YAN LI,[1] ELIZABETH M. LIVANOS,[5] MARY HIXON,[6] ALICIA WHITE,[4] DAVID BEACH,[7] AND THEA D. TLSTY[2,3,4,5,6]*

Department of Biochemistry and Biophysics,[1] Program in Molecular Biology and Biotechnology,[2] Lineberger Comprehensive Cancer Center,[3] Curriculum in Genetics and Molecular Biology,[4] Department of Pathology,[5] and Curriculum in Toxicology,[6] The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, and Howard Hughes Medical Institute, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724[7]

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Expression of viral oncoproteins results in the loss of cell cycle checkpoint control and the accumulation of chromosomal abnormalities. Expression of both human papillomavirus type 16 oncoproteins, E6 and E7, in normal human fibroblasts completely dissociates p21 and proliferating cell nuclear antigen from the quarternary cyclin–cyclin-dependent kinase (CDK) complexes present in normal cells, causes disruption of the cyclin D-CDK4 complex and replacement with a CDK4-p16 complex, and leaves binary complexes of cyclin B1-CDC2 and cyclin A-CDK2 intact. These results are identical to those observed in fully transformed cells. The expression of the individual oncoproteins dramatically affects the association of proliferating cell nuclear antigen into the complexes while leaving the total cellular levels unaltered. Expression of low-risk human papillomavirus has no effect on cyclin complexes. These findings provide evidence for the gross alteration of cyclin-CDK complexes in preneoplastic cells and links this alteration to the loss of genomic stability.

The human papillomaviruses (HPVs) are associated with tumors of the epithelial or fibroepithelial components of skin or mucosa. Numerous clinical, epidemiological, and molecular studies associate specific viral types with the formation of carcinomas of the genital and oral mucosa (68). Some HPV types (16, 18, 31, 33, and 39) are referred to as high risk because of their association with cervical carcinoma, while other types (6 and 11) associated with benign lesions are termed low risk. Two viral gene products, E6 and E7, from the high-risk virus are necessary for efficient transformation of human cells (7, 25, 41, 59). These viral oncoproteins bind a set of host cellular proteins. The HPV type 16 (HPV16) E6 gene product binds and aids in the degradation of p53 (52), while the E7 gene product binds and functionally inactivates a set of cellular proteins, including the members of the retinoblastoma protein (Rb) tumor suppressor family (10, 41). These properties of the HPV viral oncoproteins are shared by the oncoproteins of both simian virus 40 [(SV40) T antigen] and adenoviruses (E1A and E1B) (12, 56). The mechanistic effects of these protein couplings on the neoplastic process are under intense investigation.

Virally transformed cells are genomically unstable, exhibiting aneuploidy and chromosomal rearrangements (29). In studies designed to address the mechanistic basis for this observation, we found that the expression of viral oncoproteins in normal human fibroblasts (NHFs) dramatically affects cell cycle control and genomic integrity (61). We expressed the E6 and E7 viral oncoproteins from the high-risk HPV16 in an NHF strain (NHF1) that has previously been extensively characterized and lacks a detectable frequency of gene amplification. We find that NHFs expressing E6 and E7 either together (NHFE6/E7 cells) or separately (NHFE6 and NHFE7 cells) immediately alter checkpoint control. Whereas normal cells will respond to a negative growth signal (confluence, DNA damage, or metabolic inhibitors) by an arrest in cell cycle progression, E6/E7- or T-antigen-expressing cells fail to do so (50, 61). In all cases, these cells can proliferate in culture for a limited amount of time and then senesce. These oncoprotein-expressing cells have clearly lost control of genomic integrity prior to immortalization. To understand the molecular basis of checkpoint control and how it is lost in these cells, we analyzed cyclin complex perturbations in NHFs expressing viral oncoproteins.

It is now well established that the primary control of the eukaryotic cell division cycle is provided by a family of serine/threonine protein kinase complexes consisting of a catalytic subunit (cyclin-dependent kinase [CDK]) and a regulatory subunit (cyclin) (8, 43). Cyclins were originally discovered in invertebrate eggs as proteins whose abundance after fertilization oscillated during early cleavage divisions as a result of their active synthesis and abrupt proteolytic degradation at mitosis (13). Subsequently, cyclin genes have been isolated from virtually all eukaryotic species and found to constitute a multigene family (reviewed by Xiong and Beach [62]). In unicellular yeast cells, a single prototype CDK gene, *cdc2* of the fission yeast *Schizosaccharomyces pombe* or *CDC28* in the budding yeast *Saccharomyces cerevisiae*, in conjunction with the various types of cyclins controls both the G_1/S (Start) and G_2/M (mitosis) transitions. In humans and other higher eukaryotes, however, CDKs also constitute a multigene family. At least six CDK genes, CDC2 (or CDK1) and CDK2, -3, -4, -5, and -6, have been identified in human cells. Each CDK can apparently interact with any one of the multiple cyclin regulatory subunits, although sometimes favoring a particular one (reviewed by Sherr [55] and Pines [49]). In NHFs, cyclin-CDK pairs further associate with two additional proteins, proliferating cell nu-

* Corresponding author. Present address: Department of Pathology, University of California at San Francisco, San Francisco, CA 94143-0506. Phone: (415) 476-1701. Fax: (415) 476-9672.

† Present address: Department of Cardiology, Medical University of South Carolina, Charleston, SC 29412.

clear antigen (PCNA) and p21, to form a potentially large number of quaternary complexes (64, 65, 67). Upon transformation of diploid fibroblasts with the DNA tumor virus SV40 or its transforming tumor antigen, the cyclin-CDK-p21-PCNA complexes undergo drastic subunit rearrangement including dissociation of both p21 and PCNA from cyclin-CDK complexes (65). The pattern of subunit rearrangement of cyclin-CDK complexes in SV40-transformed cells is also shared in those containing adenovirus or papillomavirus oncoproteins.

To specifically address which viral oncoprotein alters the cyclin-CDK complexes and when such alterations occur, before or after the cell transformation, we analyzed in detail the subunit compositions of the complexes in mortal, nontransformed cells expressing individual viral oncoproteins that specifically inactivate either p53 or the Rb family of proteins. The biologic consequences of the expression of these viral oncoproteins that lead to alterations of cyclin-CDK complexes include defects in cell cycle checkpoint control and are seen as the accumulation of cytogenetic abnormalities as these cells propagate. These changes occur prior to cell transformation.

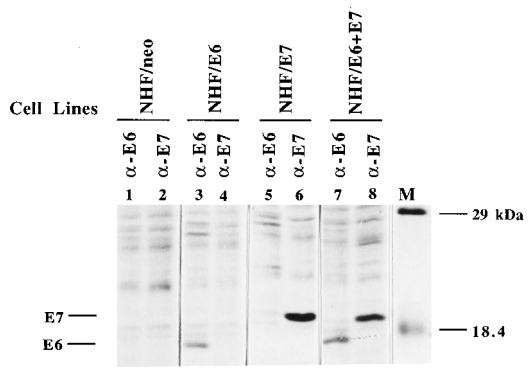

FIG. 1. Expression of HPV16 E6 and E7 genes in NHFs. [^{35}S]methionine-labeled cell lysates were prepared as described previously (65) from the indicated cell lines. Cell lysates were immunoprecipitated with an antibody specific to E6 (lanes 1, 3, 5, and 7) or E7 (lanes 2, 4, 6, and 8). The positions of E6 and E7 proteins are marked on the left, and molecular weight markers are indicated at the right.

MATERIALS AND METHODS

Cell populations and culture conditions. All cells were grown in alpha minimal essential medium without nucleosides and deoxyribonucleosides but containing glutamine and 10% dialyzed fetal bovine serum (JRH Biosciences). Primary human foreskin fibroblasts were obtained from M. Cordiero-Stone, University of North Carolina at Chapel Hill. At passage 10 or 11, cells in log-phase growth were infected with retroviral vectors containing only the neomycin resistance gene (vector LXSN) or a vector containing the neomycin gene and HPV16 open reading frames for the E6 and/or E7 proteins (LXSN16E6E7, LXSN16E6, or LXSN16E7). The vectors were a gift from Denise Galloway, Fred Hutchison Cancer Center, Seattle, Wash. Retroviral infection, population doubling time determination, and karyotypic analysis were performed as described previously (61).

Karyotypic analysis. The procedure used to obtain G-banded chromosomes was previously described (34). Fifty metaphase spreads from each cell population were analyzed, and results are expressed as a percentage of cells exhibiting the indicated karyotype.

Immunoprecipitation of viral and cellular proteins. Cells (2×10^6) were incubated in methionine- and cysteine-free Dulbecco modified Eagle medium for 1 h at 37°C and were then labeled for 3 h with 80 μCi of Tran^{35}S-Label (70% L-[^{35}S]methionine and 15% L-[^{35}S]cysteine; ICN) per ml. Incubation in 800 μl of lysis buffer (20 mM Tris [pH 7.4], 250 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 50 μg of leupeptin per ml, 30 μg of aprotinin per ml) for 10 min at 4°C was followed by centrifugation at 14,000 rpm for 30 min to remove cellular debris. Lysates were precleared by absorption with Sansorbin (Calbiochem) for 30 min on ice and microcentrifuged 15 min. Equivalent trichloroacetic acid-precipitable counts (5×10^7) of radioactive protein were immunoprecipitated with protein A-Sepharose coated with mouse anti-HPV18/16 E6 (Oncogene Science), anti-HPV16 E7 (Denise Galloway), anti-human p53 protein (p53 Ab-5; Oncogene Science), or anti-Rb antibody (RB Ab-1; Oncogene Science) for 2 h. Immune complexes were washed four times in wash buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 0.5% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS]) and twice in phosphate-buffered saline, released from the beads by heating to 95°C, and separated by electrophoresis on SDS–12% polyacrylamide gels (PAGE). Gels were fixed, treated with Amplify (Amersham), dried, and exposed to X-ray film.

Immunochemistry procedures. [^{35}S]methionine metabolic labeling and Western blotting procedures (immunoblotting) were as previously described (65). Anti-E7 antibodies were a gift of John Schiller (National Institutes of Health, Bethesda, Md.) and have been previously characterized by Androphy et al. (1) and Firzlaff et al. (16).

RESULTS

Expression of the HPV16 oncoproteins in NHFs. NHF1 cells were infected with amphotropic retroviral vectors carrying the neomycin resistance gene alone (NHF/neo cells) or in combination with HPV16-encoded oncoprotein E6 or E7 as described previously (61). Expression of both viral proteins in NHFs was examined by [^{35}S]methionine metabolic labeling as shown in Fig. 1. ^{35}S-labeled lysates were prepared and immunoprecipitated with an antibody specific to either HPV E6 or HPV E7 (1, 16). An 18-kDa protein corresponding to the expected size of HPV E6 was specifically precipitated by the anti-E6 antibody from NHFs infected with a recombinant retrovirus containing the HPV E6 gene (lane 3) or both E6 and E7 genes (lane 7). A 19-kDa protein corresponding to the expected size of E7 protein was evident in anti-E7 immunoprecipitations derived from NHFs cells infected with a retrovirus carrying the HPV E7 gene alone (lane 6) or carrying both HPV E6 and E7 genes (lane 8). Consistent with its role in facilitating the degradation of p53 (52, 60), a severe reduction in p53 protein levels was observed in cells expressing E6 (data not shown). It should be noted that these studies are done with populations of fibroblasts which express significant amounts of E6 and E7 proteins. Normally, HPV infects epithelial cells, and these genes are most likely expressed at a lower level.

Characterization of chromosome complements in HPV-infected NHFs. The oncoprotein-expressing cells were previously shown to generate drug-resistant colonies, indicating that they had the potential to be genomically unstable when challenged with a negative growth signal (61). To further elucidate the molecular basis by which the variants arise, we examined the chromosomal condition of these cells while they were grown in culture. Karyotypic stability was examined in uninfected and HPV-infected NHFs, with samples taken soon after the viral oncogene-expressing populations were selected as well as prior to their senescence in culture (Table 1). Normal cells senesce after ~72 population doublings (PDL) in culture, while the E6/E7-expressing populations have an extended life span of ~20 PDL. Cells expressing each oncoprotein individually senesce around PDL 82. Normal, diploid karyotypes were observed in the parental NHFs both at early passage and at a PDL close to senescence. Infrequently, there is a random loss of a single chromosome or the observation of a tetraploid cell. These observations are in keeping with those reported in the literature (2). At early passage after infection with the indicated oncoprotein-coding genes, the NHF/neo, NHFE6, and NHFE7 cells displayed karyotypes that were indistinguishable from those of the parental NHFs (Table 1) (61). All four populations have a basic normal karyotype, with no rearrangements detected. Aneuploidy is found in less than 10% of the cells. The karyotypes of the NHFE6/E7 cells at early passage are similar to those of the parental NHFs in that less than 10% of the cells are aneuploid, yet these cells were found to have telomeric association (defined as end-to-end fusion of chromo-

TABLE 1. Karyotypic characterization of NHFs and HPV protein-expressing fibroblasts[a]

Cell population	% of cells				
	Diploid (46 ± 2)	Tetraploid (92 ± 2)	Aneuploid	With telomeric association[b]	With translocation rearrangement
Early passage					
NHF	100	0	0	0	0
NHF/neo (PDL 40)	94	6	0	0	0
NHF16 E6/E7 (PDL 46)	96	0	4	8	6
NHFE6 (PDL 56)	100	0	0	0	0
NHFE7 (PDL 50)	96	4	0	0	0
Late passage					
NHF (PDL 70)	94	0	6	0	0
NHFE6/E7					
PDL 56	94	2	4	14	2
PDL 73	90	4	6	48	56
PDL 98	20	4	76	100	100
NHFE6					
PDL 69	94	2	4	44	66
PDL 88	20	0	80	96	98
NHFE7 (PDL 76)	100	0	0	4	0

[a] Karyotypic analysis of oncoprotein-expressing cells was compared with that of parental cells at various times during passage in culture. The data for early-passage cells are from White et al. (61).

[b] End-to-end joining of chromosomes.

somes) in 8% of the cells and rearrangements in 6% of the cells. The telomeric association figures seen were simple, each consisting of two recognizable chromosomes attached at their telomeres, with no noticeable loss or gain of chromatin at the junctions.

At late passage, the different effects of E6 and E7 are revealed. The E7-expressing cell population retained a normal, diploid karyotype, even at two PDL from senescence (Table 1). In contrast, upon passage in tissue culture (with no overt introduction of damage), the E6/E7- and E6-expressing cells have similar, highly aberrant karyotypes. The NHFE6/E7 and NHFE6 cell populations lost any semblance of a diploid chromosome karyotype; mean chromosome count for the NHFE6/E7 cells was 68 (range, 37 to >100), while the mean chromosome count for the NHFE6 cells was 69 (range, 40 to >100); these means are underestimates since cells with more than 100 chromosomes were tallied as having exactly 100 chromosomes. Virtually all of the cells (>95%) in these two populations have complex chromosome rearrangements and telomeric associations. Often the chromosomes involved could not be recognized. Some telomeric association figures consisted of strings of several chromosomes attached end to end, and some had extra or missing material at their junctions. Some of the dicentrics figures observed were the result of a single chromosome duplicating one of its own arms and centromere region rather than the result of telomeric association.

To verify that the increased aneuploidy and rearrangements observed in the later-passage E6/E7-expressing cells was not simply a reflection of their increased number of PDL (because of extended life span), we also examined their karyotypes at an intermediate PDL that corresponded to the samplings of the E6- or E7-expressing populations prior to senescence. As noted in Table 1, increased rearrangement and telomeric association were also observed in the NHFE6/E7 cells at that point in time as well. At earlier passage, usually the telomeric association and rearrangements were simple, involving only two chromosomes at a time, and were not totally random. In the NHFE6/E7 cell population at intermediate passage, chromosomes 12p and X were often involved. The types of chromosomal abnormalities that were observed as the NHFE6/E7 cell population progressed in culture followed a discernible order (see Discussion).

Cyclin-CDK complexes in the parental cells. Our previous studies have shown that NHFs have checkpoint responses that are intact and will arrest in the G_1 and G_2 phases of the cell cycle when exposed to a negative growth signal or DNA damage (references 34 and 61 and unpublished observations). It is hypothesized that these pauses in cell cycle progression prevent the use of damaged DNA for replication and its segregation, preventing the formation of chromosomal abnormalities. Deviations from this pattern are seen in NHFs expressing HPV16 viral oncoproteins (61). These cells fail to arrest when challenged with a negative growth signal. To implicate the cyclin involvement in these alterations in cell cycle control, we examined the complexes of cyclins D1, A, and B1 in the oncoprotein-expressing cells. Since rearrangement of quaternary complexes has been found to be extensive in fully transformed cells, we sought to examine these complexes in their nontransformed progenitors. To ensure that any changes were regulatory in nature, rather than a product of genomic alteration, only cyclin complexes from early-passage cells (passages 42 to 48), which exhibited a normal karyotype, were examined.

First, we characterized the cyclin-CDK complexes in NHFs and NHF/neo cells for comparison with previous normal cell populations (Fig. 2). [^{35}S]methionine-labeled cell lysates derived from NHFs and NHF/neo cells were immunoprecipitated with a battery of six antibodies (anti-human CDC2, CDK2, CDK4, cyclin A, cyclin B1, and cyclin D1). In both cell populations, association of p21 with each of the cyclin and CDK complexes was readily observable, as in other NHF cell lines previously characterized (63, 67). However, the intensity of the PCNA protein band in cyclin-CDK complexes, as determined by the metabolic labeling, is much lower than that observed in other NHF lines. To address this issue, we performed immunoblotting coupled with immunoprecipitation (Fig. 3). PCNA proteins were readily detected by Western blotting in all cyclin and CDK complexes of normal cells with the exception of CDC2 complexes, which contained a barely detectable level. We have observed somewhat similar phenomena before and do not yet fully understand this apparent experimental discrepancy between metabolic labeling and Western blotting between the different NHF populations. For example, the levels of PCNA protein present in both cyclin D1 and CDK4 complexes are similar in NHFs, NHF/neo cells, and NHFE7 cells, as determined by Western blotting (compare lanes 4, 5, and 7 in Fig. 3), but the intensity of the [^{35}S]methionine-labeled PCNA band is much higher in NHFE7 cells (see Fig. 5) than in NHFs or NHF/neo cells. A possible explanation for this is that the rate of PCNA synthesis and its recruitment into cyclin-CDK complexes differ in different types of cells.

Cyclin-CDK complexes in NHFE6 cells. As shown below, expression of HPV16 E6 dramatically decreases the level of p21 mRNA (see Fig. 8). We examined the association of p21 protein with the cyclin complexes in these cells. Interestingly, the amount of p21 protein present in the cyclin D1- and cyclin A-CDK complexes (as determined by metabolic labeling) is similar to that seen in NHFs and NHF/neo cells (Fig. 4). However, no p21 protein was detected in [^{35}S]methionine-labeled anti-CDC2 and anti-cyclin B1 immunoprecipitates (Fig. 4, lanes 1 and 7). CDC2 and cyclin B1 are the major catalytic (CDC2) and regulatory (cyclin B1) partners of each other. Together, these results suggest that p21 has different

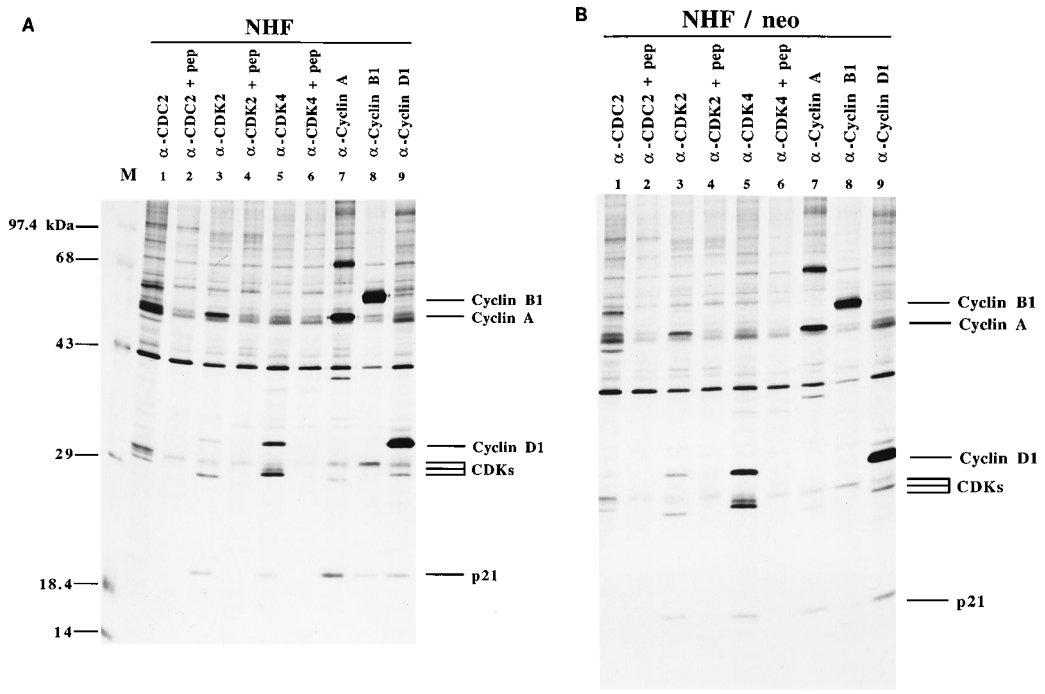

FIG. 2. Cyclin-CDK complexes in NHFs and NHF/neo cells. [^{35}S]methionine-labeled cell lysates were prepared from NHFs (strain NHF1) or NHF/neo cells and immunoprecipitated with one of six antisera, each specific to a cyclin or CDK, with or without prior incubation with a competing antigen peptide (pep) (as indicated above each lane) and resolved by SDS-PAGE. The relative positions of the molecular weight markers and proteins whose identities have been established are marked.

association affinities for different cyclin-CDK complexes and that the reduced level of p21 may selectively dissociate p21 from specific cyclin-CDK complexes.

PCNA protein was not seen in any [^{35}S]methionine-labeled cyclin or CDK immunoprecipitates derived from NHFE6 cells (Fig. 4). The profound dissociation of PCNA from cyclin-CDK complexes was further confirmed by immunoblotting (Fig. 3). The level of total PCNA protein in NHFE6 cells is similar to that in NHFs or NHF/neo cells (compare lane 6 with lanes 4 and 5), as determined by immunoblotting of the total cell lysate, yet this protein is not found in complexed form. The dissociation of PCNA protein from cyclin-CDK complexes is specific to the oncogenic forms of HPV, since a normal level of PCNA protein was detected in NHFs expressing the nononcogenic form of E6 from HPV6, and the PCNA is found in association with the cyclin complexes, albeit to a slightly diminished degree (Fig. 3). The major biological function known to associate with the oncogenic E6 from high-risk HPV16 but not low-risk HPV6 is binding to and promoting the in vitro degradation of p53 (52, 60). Thus, this finding suggests that in addition to regulating the transcription of p21 (see below), p53 may also indirectly regulate the association of PCNA with cyclin-CDK complexes.

Cyclin-CDK complexes in NHFE7 cells. We next investigated the cyclin-CDK complexes in NHFE7 cells. Unlike the cells expressing HPV16 E6, quaternary complexes are very much intact in cells expressing the E7 oncoprotein. Indeed, the intensities of both p21 and PCNA as determined by [^{35}S]methionine-labeling are considerably higher than those seen in NHFs and NHF/neo cells (Fig. 5). The higher levels of PCNA in all cyclin-CDK complexes as detected by [^{35}S]methionine labeling were further confirmed by immunoblotting experiments (Fig. 3). While the total amount of PCNA protein in NHFE7 cells (Fig. 3, lane 7) is similar to that in NHFs or NHF/neo cells (lanes 4 and 5) and slightly higher than that in NHFE6 cells (lane 6), PCNA protein is present in all cyclin and CDK complexes at levels either similar to (cyclin D1 and CDK4) or considerably higher than (cyclin B1, cyclin A, CDC2, and CDK2) levels in NHFs and NHF/neo cells. The increased association of PCNA with these cyclin-kinase complexes is specific to E7 of HPV16 since the low-risk form does not show a significant change in the PCNA binding (lane 2). Increased association of both p21 and PCNA with cyclin-CDK complexes in cells expressing E7 proteins points to the possibility that in addition to being regulated by p53, the cell cycle machinery is regulated by a pathway involving the Rb family of proteins (Rb, p107, p130, etc.). These changes have previously been associated with altered checkpoint control (61).

Cyclin-CDK complexes in NHFE6/E7 cells. The different effects of E6 and E7 on the genomic stability and cyclin-CDK complexes prompted us to further analyze the cyclin-CDK complexes in NHFE6/E7 cells. As in cells expressing E6 alone, no PCNA was detected, either by [^{35}S]methionine labeling (Fig. 6) or by immunoblotting (Fig. 3, lane 8), in any cyclin-CDK immunocomplexes derived from NHFE6/E7 cells (except perhaps a trace amount of PCNA in CDK2 immunoprecipitates). However, expression of E6 together with E7 had apparently different effects on the association of p21 with cyclin-CDK complexes than expression of either E6 or E7 alone. p21 protein is totally absent from all cyclin-CDK complexes, as determined by [^{35}S]methionine labeling. Therefore, it appears that while the expression of E6 alone is sufficient to dissociate PCNA from cyclin-CDK complexes, expression of both E6 and E7 is required for the complete absence of p21 from cyclin-CDK complexes. The configuration of cyclin-CDK complexes in NHFE6/E7 cells is very much the same as that in cells fully transformed by SV40 (VA13 or CT10 cells), adenoviruses (293 cells), or papillomaviruses (HeLa cells [65]). The one excep-

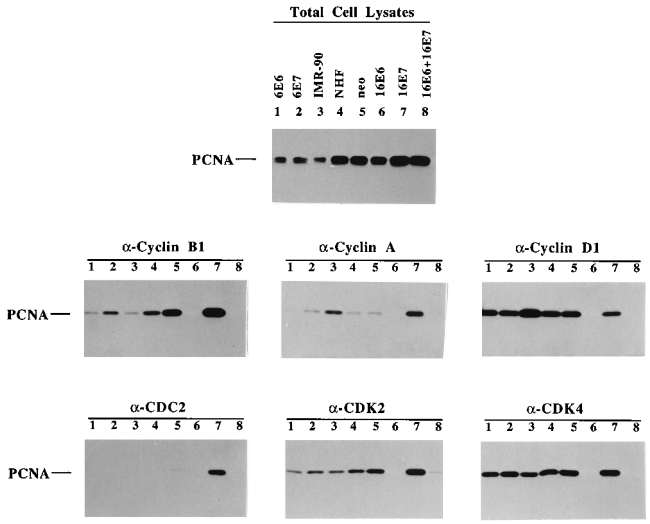

FIG. 3. Immunoblotting analysis of PCNA association with cyclin-CDK complexes. Total cell lysates were prepared from two NHF cell lines (IMR-90 and NHF1; lanes 3 and 4) and NHF-1 cells transfected with retroviral vectors containing the neomycin resistance gene (neo; lane 5), low-risk HPV6 E6 oncogene (lane 1), HPV6 E7 oncogene (lane 2), high-risk HPV16 E6 oncogene (lane 6), HPV16 E7 oncogene (lane 7), and HPV16 E6 together with HPV16 E7 (lane 8). These cell lysates were immunoprecipitated with one of six antisera, each specific to a cyclin or CDK (as indicated above each set of lanes), and resolved by SDS-PAGE. In the panel at the top, total lysates from each cell line were resolved by SDS-PAGE without immunoprecipitation. Proteins resolved on an SDS-polyacrylamide gel were electrotransferred to a nitrocellulose filter, and the blots were probed with a monoclonal antibody specific to PCNA.

tion is cyclin A-CDK2. In fully transformed cells, this complex associates with the p19 protein, but in E6/E7-expressing cells, it is not detected. These results indicate that the subunit rearrangement of cyclin-CDK complexes previously observed in fully transformed cells occurred before rather than after neoplastic transformation, suggesting that such alterations of cell cycle machinery may be a prerequisite for, not a consequence of, cellular transformation.

Cyclin D-CDK4 complexes. Unlike cyclin B1-CDC2 and cyclin A-CDK2 complexes, whose binary association is not affected in fully transformed cells, cyclin D-CDK4 association is totally disrupted in transformed cells in addition to the dissociation of p21 and PCNA (65). This phenomenon was further investigated by immunoblotting lysates from NHFs expressing individual HPV oncoproteins. Anti-cyclin D1 and anti-CDK4 immunoprecipitates derived from the panel of cells were probed by both anti-cyclin D1 and anti-CDK4 antibodies simultaneously to examine their levels and to determine the association between them (Fig. 7). It is evident from this experiment that the expression of HPV16 E6 alone not only dissociates p21 and PCNA from cyclin D1 and CDK4 complexes but also drastically disrupts the binary association of cyclin D1-CDK4. Only a very small fraction of the cyclin D1 and CDK4 is found associated with each other in cells expressing E6. The slight discrepancy between immunoblotting (Fig. 7) and metabolic labeling (Fig. 4, lane 4) may reflect more sensitive detection by the latter assay because of the very short half-life of cyclin D1 (~30 min [54]). Expression of E7 has little effect on the cyclin D1-CDK4 complex, but when E7 is expressed together with E6, it appears that the disruption was more complete (Fig. 7, lanes 5 and 12; Fig. 6, lanes 4 and 7). The effect of E6 on cyclin D1-CDK4 complexes is specific to the oncogenic forms of HPV, since expression of nononcogenic HPV6 E6 did not show any effect on their association. Together with the observations that expression of HPV16 E6 down regulates the level of p21 mRNA and also disrupts the PCNA association with cyclin-CDK complexes, it is possible that the cyclin D-CDK4 complexes are stabilized by p21 and PCNA association. Experiments with cyclin D1 and CDK2 yielded similar results (data not shown).

p16-CDK4 complexes. In SV40-, adenovirus-, and papillomavirus-transformed cells, CDK4 proteins dissociate from cyclin D1 and exclusively associate with p16 (65). This observation is in agreement with the report of absence of cyclin D protein and cyclin D-CDK complexes in cells lacking pRb (4, 33, 46, 51). Consistent with previous reports, expression of either E6 or E7 alone promotes the formation of p16-CDK4 complexes at least 10-fold above that seen in parental cells (compare lanes 4 and 5 of Fig. 4 and 5 with lanes 5 and 6 of Fig. 2A and B). In cells expressing both E6 and E7, all CDK4 is associated with p16, and the level of this binary complex was significantly higher than with either oncoprotein alone, further reinforcing the notion that both p53 and Rb may negatively regulate the expression of p16 (33). Increased levels of p16 in fully transformed or E7-expressing cells result in part from the release of transcriptional repression of the p16 gene by Rb (33).

Expression of p21 mRNA in HPV-infected NHFs. Using the recently isolated human p21 cDNA probe (63), we investigated the steady-state levels of p21 mRNA in NHFs as well as in NHFs expressing HPV oncoproteins (Fig. 8). Total RNAs prepared from the panel of cells were subjected to Northern (RNA) analysis and hybridized with a full-length p21 cDNA probe. Strikingly, expression of E6 dramatically reduced the

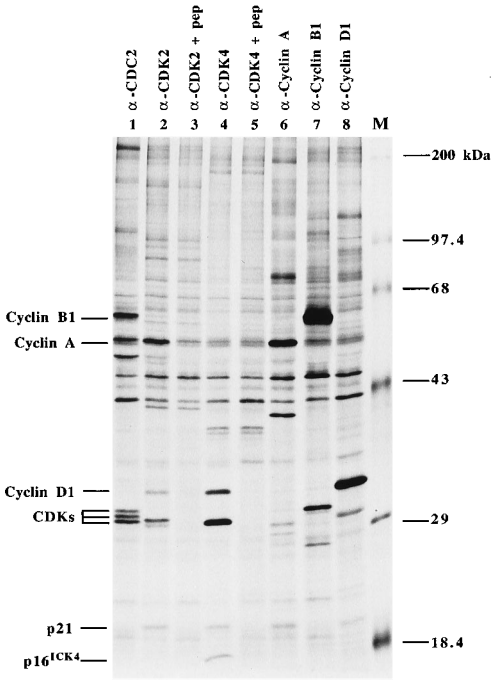

FIG. 4. Subunit rearrangement of cyclin-CDK complexes in NHFE6 cells. [^{35}S]methionine-labeled cell lysates were prepared from NHFE6 cells. Labeled cell lysates were immunoprecipitated with six antisera, each specific to a cyclin or CDK, with or without prior incubation with a competing antigen peptide (pep) (as indicated above each lane) and resolved by SDS-PAGE. The relative positions of the molecular weight markers and proteins whose identities have been established are marked.

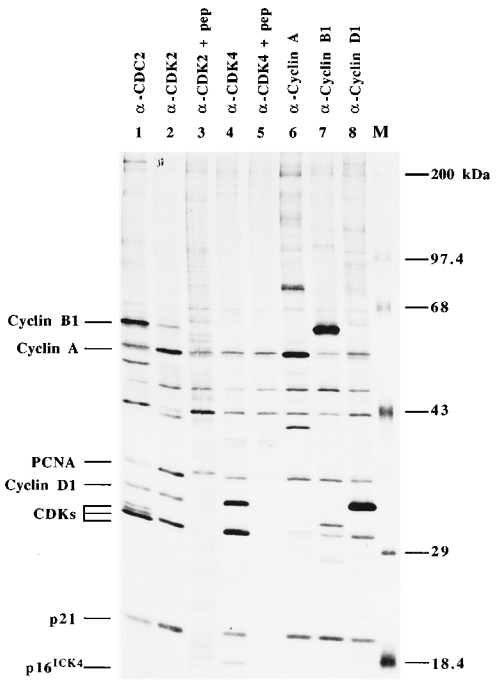

FIG. 5. Subunit rearrangement of cyclin-CDK complexes in NHFE7 cells. [^{35}S]methionine-labeled cell lysates were prepared from NHFE7 cells. Labeled cell lysates were immunoprecipitated with six antisera, each specific to a cyclin or CDK, with or without prior incubation with a competing antigen peptide (pep) (as indicated above each lane) and resolved by SDS-PAGE. The relative positions of the molecular weight markers and proteins whose identities have been established are marked.

level of p21 mRNA (Fig. 8, lane 3). This effect is specific to the oncogenic form of HPV16 E6 since expression of nononcogenic HPV6 E6 had little effect on the level of p21 mRNA (lane 6), indicating that p53 may directly regulate the transcription of p21. These results are entirely consistent with a report that p21 encodes a major transcript whose transcription is induced by wild-type but not mutant p53 (11). Expression of E7 has little effect on the level of p21 mRNA. Similarly, low levels of p21 mRNA are seen in cells expressing E6 alone or E6 plus E7, yet p21 protein is associated with cyclin-CDK complexes (D1 and A) in E6-expressing cells but not E6/E7-expressing cells; this result suggests that E7 activity can also affect the association of p21 with cyclin-CDK complexes. Cellular proteins such as Rb, p107, and p130 may be involved in the posttranscriptional modification or localization of p21.

DISCUSSION

The origin and development of human tumors begin at the molecular level and involve a complex multistep process. Correlated with this process is the loss of genomic integrity, a hallmark of neoplastic cells (15, 18, 26, 44). The dependence of cell cycle progression on genomic integrity has been described as checkpoint control (24). Many recent efforts in the study of genomic integrity have focused on elucidating the molecular pathway(s) of checkpoint control in normal cells and how such a pathway(s) is altered or becomes defective during cellular transformation. In human fibroblasts fully transformed by a variety of viral oncoproteins, including E6 and E7, the primary regulators of eukaryotic cell cycle, CDKs, are grossly altered (65). We have demonstrated that in preneoplastic mortal cells, checkpoint control is disrupted by divergent pathways by expression of either the E6 or E7 viral oncoprotein of HPV (61) or T antigen of SV40 (50). We have now further analyzed the subunit composition of CDKs in preneoplastic mortal cells expressing HPV E6 and E7 in an attempt to resolve how the viral oncoproteins alter cell cycle machinery and observe the effects on genomic integrity.

Alteration of cyclin-CDK complexes in preneoplastic cells. In NHFs, CDKs predominantly exist as quaternary complexes, each consisting of a cyclin, a CDK, PCNA, and p21. Upon cellular transformation by DNA tumor viruses, the cyclin-CDK-p21-PCNA complexes undergo a dramatic subunit rearrangement (65), indicating that a common mechanism may be used by these different tumor viruses in altering normal cell cycle control. However, since those experiments were carried out in fully transformed cells, it is not possible to tell when the alteration occurred, before or after the cell transformation. Using NHFs expressing viral oncoproteins E6 and E7 (61), we demonstrate here that cyclin-CDK complexes are grossly altered in preneoplastic mortal cells (Fig. 9). Analyses performed in early- and mid-passage cells yielded identical results. The subunit composition of all cyclin-CDK complexes that we have examined is the same in NHFE6/E7 cells as that observed in fibroblast cells fully transformed by SV40, adenoviruses, or papillomaviruses (Fig. 6 and reference 65). Specifically, no p21 or PCNA is associated with the various cyclin-CDK complexes, and CDK4 becomes exclusively associated with the kinase inhibitor p16 (53), with concomitant disruption of cyclin D1-CDK4 binary association. Hence, the subunit rearrangement of cyclin-CDK complexes is a direct effect of viral oncoprotein expression rather than a consequence of cellular transformation. We suggest from these observations that alteration of cyclin-CDK complexes is a major target of viral oncoprotein function and a prerequisite for cellular transformation.

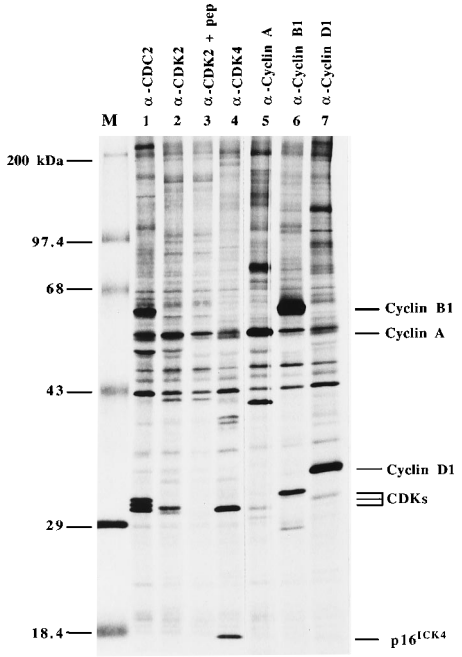

FIG. 6. Subunit rearrangement of cyclin-CDK complexes in NHFE6/E7 cells. [^{35}S]methionine-labeled cell lysates were prepared from NHFE6/E7 cells. Labeled cell lysates were immunoprecipitated with six antisera, each specific to a cyclin or CDK, with or without prior incubation with a competing antigen peptide (pep) (as indicated above each lane) and resolved by SDS-PAGE. The relative positions of the molecular weight markers and proteins whose identities have been established are marked.

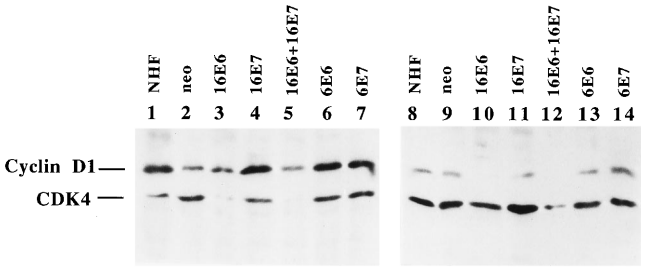

FIG. 7. Immunoblotting analysis of cyclin D1-CDK4 complexes. Total cell lysates were prepared from NHF1 cells (lanes 1 and 8) and NHF-1 cells transfected with retroviral vectors containing the neomycin resistance gene (neo; lanes 2 and 9), high-risk HPV16 E6 oncogene (lanes 3 and 10), HPV16 E7 oncogene (lanes 4 and 11), and HPV16 E6 and E7 oncogenes (lanes 5 and 12), low-risk HPV6 E6 oncogene (lanes 6 and 13), and low-risk HPV6 E7 oncogene (lanes 7 and 14). Lysates were immunoprecipitated with either an anti-cyclin D1 (lanes 1 to 7) or anti-CDK4 (lanes 8 to 14) antiserum. Immunoprecipitates resolved on an SDS-polyacrylamide gel were electrotransferred to a nitrocellulose filter, and the membranes were probed with a mixture of antisera against cyclin D1 and CDK4.

Alteration of cyclin-CDK complexes and loss of genomic integrity. Although ubiquitous, the presence of HPV16 or HPV18 is not sufficient to trigger malignant progression. The virus may be present with a long latency, and only rarely does progression of genital lesions into carcinomas occur (68). The so-called high-risk HPVs are carried in an episomal state in benign tumors and are found in an integrated state in malignant lesions (9, 47). This integration event is accompanied by a change in transcription activity and pattern (3, 32). In the episomal state, an entire spectrum of viral proteins used for replication, capsid formation, etc., is expressed, whereas in the integrated state, the E6 and E7 viral oncogenic products become the dominant transcripts (9, 47). Our studies have documented the effect of the expression of these oncoproteins on checkpoint control and genomic stability (61).

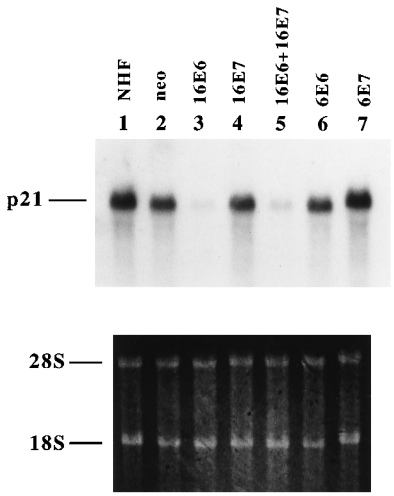

FIG. 8. Expression of p21 mRNA in fibroblasts expressing viral oncoproteins. Total RNA was prepared from NHFs (lane 1) and NHFs infected with amphotropic retroviruses carrying a neomycin resistance marker (neo; lane 2), genes encoding high-risk HPV16 E6 (lane 3), E7 (lane 4), and E6 plus E7 (lane 5), and genes encoding low-risk HPV6 E6 (lane 6) and E7 (lane 7). RNA samples were resolved on a 1% agarose gel (lower panel) and transferred to a nitrocellulose filter. The membrane was hybridized with a probe derived from the p21 cDNA (top panel). The positions of p21 mRNA and 18S and 28S rRNAs are indicated at the left.

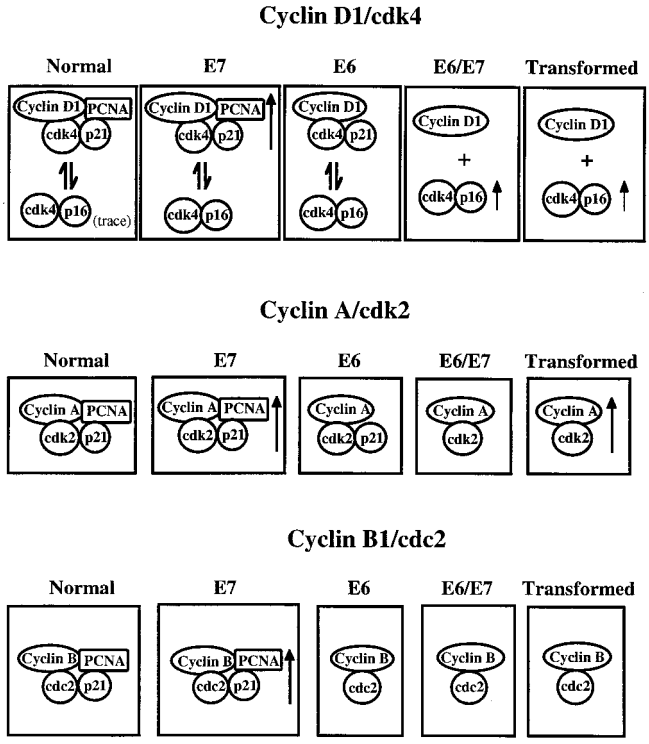

FIG. 9. Summary of proteins associated with cyclins and CDKs. Primary associations are depicted in cartoon fashion. Arrows located beside complexes indicate a change in the quantity of the complex detected by the methods described in the text. Data for the fully transformed cells were taken from Xiong et al. (65).

Genetic instability in the HPV oncoprotein-expressing cells is evident in the absence (Table 1) as well as in the presence (61) of *N*-phosphonoacetyl-L-aspartate selection. Yet, while expression of each oncoprotein individually allows the generation of drug resistance (i.e., genomic instability), the types of changes that occur differ dramatically. Cells that contain compromised p53 function (cells expressing E6 alone or E6 and E7) survive, with chromosomes that have evidently been broken and rejoined. Translocations, amplifications, and deletions are evident (Table 1 and reference 61). These rearrangements increase dramatically as the cells are passaged beyond the threshold of senescence. In contrast, in cells that retain p53 function but are altered in the Rb family of proteins (cells that are expressing E7), the breakage abnormalities are not observed. One possibility for this phenomenon is that physical damage to the genome, such as DNA breaks postulated in the bridge-breakage-fusion cycles (favored as a mechanism by which gene amplification occurs), trigger p53 activation, and these cells are cleared from the population. Under adverse conditions (i.e., lack of metabolites or the presence of DNA breaks), these cells commit suicide rather than deal with unfavorable consequences. Whole chromosome anomalies, arising from nondisjunction, may not trigger p53 activity and are thereby permitted. Either the breaks do not occur in the first place or the cells that contain them selectively die. Either of these processes would result in the relatively normal karyotype seen in the late-passage E7-expressing cells. Only whole chromosome changes (such as polyploidy or aneuploidy) are compatible with viability in these cells. As demonstrated previously (61), the sole mechanism of drug resistance in the E7-expressing cells is an increase in the whole chromosome 2. It should be

noted that aneuploidy is the most frequent genomic change found in transformed cells.

Effects of E6 on cyclin-CDK complexes. Using NHFs expressing only the E6 or E7 oncoprotein, we were able to address how the individual oncogenic activity of each affects and activates the cell cycle machinery. Expression of high-risk HPV16 E6, but not low-risk HPV6 E6, dramatically decreases the level of p21 mRNA (Fig. 8). Significant reduction of p21 mRNA was also observed recently in a different line of NHF expressing HPV16 E6 (33). As only HPV16 E6 binds to and targets the degradation of p53 (6, 28, 52), these results provide further support for the current model that p21 is transcriptionally regulated (at least in part) by p53 (11). p21 has recently been shown to encode a potent inhibitor of CDKs (20, 22, 63). Thus, stimulation of reentry into the cell cycle by DNA tumor viruses can be partially explained by the decrease in the level of p21's inhibitory function.

Expression of E6 alone did not completely abolish the expression of p21, presumably because of incomplete degradation and inactivation of p53. p21 protein was still present in many of the cyclin-CDK complexes. However, the limited amount of p21 is likely to associate with cyclins and CDKs at a reduced level, as evidenced by the selective dissociation of p21 from the cyclin-CDK complexes to which p21 has the lowest affinity, such as CDC2 and cyclin B1 (Fig. 4). In addition to the reduced level and association of p21 with cyclin-CDK complexes, PCNA was not present in any of cyclin-CDK complexes (Fig. 4). Since we never observed association of PCNA with cyclin and CDK without p21, these findings suggest that the association of PCNA with cyclin-CDK complexes may be bridged by p21. This is consistent with the existence of two functionally distinct domains of the p21 molecule, a CDK-inhibitory domain located in the amino-terminal half and a PCNA-binding domain in the carboxyl-terminal half (5, 36, 42, 58). Taken together, the data suggest that expression of oncogenic E6 not only impairs the p53 activation of p21 expression but also completely abolishes the as yet unidentified function of PCNA in regulating the activity of CDKs. Such drastic alteration in the regulation of cell cycle kinases correlates with the karyotypic analysis that shows loss of genetic stability in E6-expressing cells (Table 1; see above).

Effects of E7 on cyclin-CDK complexes. Conversely, expression of E7 alone neither affected the expression of p21 nor altered the subunit composition of cyclin-CDK quaternary complexes. Unexpectedly, E7 appears to somehow increase the association of p21 and PCNA with cyclin-CDK complexes. A considerably higher amount of PCNA was detected in cyclin B1-CDC2 and cyclin A-CDK2 complexes in E7-expressing cells than in normal cells (Fig. 3). This effect is specific to the oncogenic form of E7, since the expression of low-risk E7 of HPV6 did not show the same effect (Fig. 3). As the levels of p21 and PCNA protein measured in E7-expressing cells are similar to those in normal cells, we do not yet know the mechanism by which E7 expression increases the association of p21 and PCNA with cyclin-CDK complexes. One possibility is that expression of E7 can directly or indirectly posttranslationally modify p21. Recent reports have demonstrated an independent association between p21 and PCNA (17, 57). The reports demonstrate that direct binding of p21 to PCNA inhibits DNA replicative synthesis. Our results show that in cells with a functionally inactivated p53, PCNA is no longer associated with the cyclin quaternary complex (Fig. 3, 4, 6, and 9). This finding suggests that p21 is not the sole determinant of PCNA association in cyclin-CDK complexes. The finding that quaternary cyclin-CDK complexes are elevated but remain intact in E7-expressing cells suggests that different aspects of genomic integrity are monitored by different cellular components. The alteration provoked by the expression of E7 in normal cells is compatible with the generation of aneuploidy but not amplification (reference 61 and Table 1; also see discussion above).

Interestingly, coexpression of E6 and E7 leads to further alteration of CDKs. p21 is completely absent from all cyclin-CDK complexes, in addition to the complete dissociation of PCNA (Fig. 6). Since expression of E7 alone did not affect the level of p21 message, this finding reinforces the suggestion that E7 activity may posttranscriptionally regulate p21. This finding further suggests that the requirement of both oncogenic activities for cellular transformation may be due to a requirement for both activities in order to completely dissociate p21 and PCNA from cyclin-CDK complexes.

No effects with low-risk viral oncoproteins. It should be noted that all alterations of cyclin-CDK complexes described here are specific to the oncogenic form of viral proteins that were derived from high-risk types of HPV. Expression of neither E6 nor E7 derived from low-risk HPV has any effect on the expression of individual components or formation of cyclin-CDK-p21-PCNA complexes. This is particularly evident in the case of p21 mRNA expression, in which case HPV16 E6 dramatically reduced the level of p21 mRNA whereas expression of HPV6 E6 had no effect (Fig. 8). Increased association of PCNA with cyclin-CDK complexes was also seen in cells expressing HPV16 E7 but not in cells expressing HPV6 E7 (Fig. 3). Studies of human cell transformation have shown that HPV6 does not immortalize primary human epithelial cells (21, 48). There is no evidence reported of complex formation between HPV6 E6 and cellular p53, and the affinity of HPV6 E7 protein for cellular Rb is approximately 10-fold lower than the affinity of HPV 16E7 for Rb (19, 40). In the presence of *N*-phosphonoacetyl-L-aspartate, the NHFs separately expressing the E6 and E7 proteins of the low-risk HPV exhibited a phenotype typical of uninfected NHFs; they retain cell cycle checkpoint control and fail to produce drug-resistant colonies. These observations further support the link between the alteration of cyclin-CDK complexes, loss of genomic integrity, and neoplastic transformation.

Cyclin D complexes. Since its discovery, the function of cyclin D1 has been the focus of many studies and still remains elusive. Among all cell cycle regulators, cyclin D1 is the most strongly implicated in oncogenesis (see reviews by Lammie and Peters [31] and Motokura and Arnold [39]; 27, 35). D cyclins differ from other members of cyclin family in several distinct ways. They associate with virtually all CDKs, albeit with a strong preference with CDK4. Expression of individual members of the D cyclins is cell type and tissue specific, and the level of cyclin D, both message and protein, does not significantly oscillate during the cell cycle in many cell types as with other cyclins. The cyclin D-associated kinase activity clearly has a substrate specificity different from those of other cyclins, with the majority of activity for cyclin D-CDK4/6 being detected with the Rb protein and its relatives (14, 30, 37, 38).

Analysis of cyclin D complexes in cells expressing the individual HPV oncogenes revealed further differences between D cyclins and other cyclins. First, identical to fully transformed cells, the cyclin D1-CDK4 and cyclin D1-CDK2 binary associations are dramatically reduced in cells expressing E6 and totally disrupted in cells expressing E6 together with E7, while other cyclin-CDK binary associations (e.g., cyclin B1-CDC2 and cyclin A-CDK2) remain largely intact in both cell types (Fig. 6). We interpret the reduction of cyclin D-CDK binary association in E6-expressing cells as the result of decreased expression of p21 that has been implicated in stabilizing cyclin-CDK complexes (23, 55). As in the fully transformed cells (54),

cyclin D-CDK complexes are further reduced to nearly complete absence in cells expressing both the E6 and E7 viral oncoproteins, most likely as the result of the combined effects of reduced p21 and activated p16 gene expression. We have recently demonstrated that the transcription of p16 is repressed by Rb, forming a regulatory feedback loop involving Rb, CDKs, and p16 (33). According to this mechanism, phosphorylation of Rb by cyclin D-CDK4 or cyclin D-CDK6 results in the release of a transcription factor (e.g., E2F-1) which is inactive when bound to hypophosphorylated Rb. Disassociation of the RB-transcription factor complex, through Rb phosphorylation by CDK or inactivation by viral oncoproteins such as HPV16 E7, would then activate p16 transcription. As levels of p16 mRNA increase, increased binding of p16 to CDK4 and CDK6 would prevent cyclin D from binding to CDK4 and CDK6 and subsequent degradation. In addition to phosphorylating and controlling the activity of Rb and the related p107 and p130 proteins, cyclin D may be involved in the regulation of such additional checkpoints as PCNA-dependent DNA repair and replicative synthesis (45). Therefore, complete dissociation and thus functional inactivation of cyclin D-CDK complexes by the expression of E6 together with E7 may also impair other, as yet unidentified cyclin D-mediated growth control pathways.

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The first two authors contributed equally to this work.

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